

The University of Chicago

**THE
VARIANT AND FILTERABLE FORMS
OF CERTAIN GREEN-PRODUCING
STREPTOCOCCI**

**A DISSERTATION SUBMITTED TO THE
FACULTY OF THE DIVISION OF THE BIO-
LOGICAL SCIENCES IN CANDIDACY FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY**

**DEPARTMENT OF HYGIENE AND BACTERIOLOGY
1933**

**By
RUTH ALDEN McKINNEY**

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THE VARIANT AND FILTERABLE FORMS OF CERTAIN GREEN-PRODUCING STREPTOCOCCI¹

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Interest in the problem of bacterial variation during the last decade has brought the heterogeneous group of green-producing streptococci under scrutiny. Studies of the reciprocal relationship between colony form (rough or smooth) and virulence, antigenic structure, opsonic index, sensitizing property, varying cataphoretic potentials and the production of filterable forms have brought forward much new knowledge of variation within this group of organisms. In many of these reports the occurrence of morphologically variant cells is mentioned as an incidental observation. Such variant cells are often diptheroids, frequently diplococci and occasionally swollen, nodular rods. The origin of the distinct cell types, their essential properties, their interrelationships, and their fate have not been clearly recorded.

Pleomorphic green-producing streptococci upon primary isolation and after extended cultivation exhibit chains of flattened diplococci of greatly varying sizes, huge coccal and constricted forms and spindle-shaped rods. The unstable form of the organisms suggests that such pleomorphic strains may be especially suitable for the study of the origin of variant cells, their distinctive properties and relationships, as well as of the occurrence of filterable forms in streptococci.

Sixty-one strains of highly pleomorphic green-producing streptococci were isolated from human throats during the early,

¹ The study was aided by a generous grant from the Influenza Commission of the Metropolitan Life Insurance Company.

active and convalescent stages of acute upper respiratory infections occurring in Chicago from December, 1928, to February, 1931. One exceptional strain, No. 42X, was isolated from the blood culture of a patient during the early stages of influenzal infection. The characteristics of this strain (with its smooth and rough substrains) have been described at length in a previous report (Falk, Harrison, McKinney and Stuppy, 1931); therefore it has been used as a standard of comparison for the remaining strains.

NATURAL HISTORY OF THE STREPTOCOCCAL STRAINS

Swabs passed over the reddened fauces, tonsils and pharynx were streaked on plates of heated blood agar and hemolyzed blood agar.² The latter transparent medium was preferred since it clearly revealed the colonial structure by *transmitted light* which simplified greatly the technic of isolation. The predominating organisms on the plates were green-producing streptococci of many colonial types. On every plate on which pleomorphic streptococci were found, another organism growing in gray mucoid colonies or in wrinkled colonies was conspicuously present. This was a Gram-negative diplococcus which in cultural reactions did not conform wholly to the Neisserian types listed by Bergey. This is no new observation (Jordan, 1927; Cooper, 1929; Traut and Herrold, 1930; Falk et al., 1931; Coburn, 1931); whether it represents a chance association, a "satellite phenomenon," or a commensal relationship has not been determined.

The colonies of pleomorphic streptococci on all solid mediums were extremely minute. Unfortunately, they possessed no characteristic colonial form by which the strains could be certainly recognized in a mixed streptococcal flora. All gradations of colonies ranging from definitely smooth colonies to markedly rough colonies were found on the original plates. The smooth colonies were very minute, round, raised and glassy yellow; the intermediate colonies were somewhat larger than the smooth colonies, round, raised and glassy yellow surrounded by a whitish

² Hemolyzed blood agar: 2 per cent sheep blood laked in water added to veal infusion agar, pH 7.8, at 60° to 70°C., giving a clear, reddish-brown agar.

halo of streptococci growing in a single plane (pl. A, fig. 1). The halo was the first visible evidence of dissociation from the smooth to the intermediate state. The roughest colonies were flat, gray, coarsely granular, with or without a central depression and irregular edge (pl. A, fig. 2).

Occasionally on the primary plates rough, grooved colonies were found which had little effect upon the blood but which left their exact impress in the agar—a mirror image of the grooved or capitate form of the exposed surface of the colony. The organisms in such colonies were swollen rods and huge cocci. At times colonies which powerfully yellow-bleached the medium were found to contain Gram-positive diplococci. The solid blood medium immediately surrounding the colonies at first was rendered green by the growing colonies (6 to 8 hours); after twenty-four hours this area had a light yellow or “bleached” appearance. Diphtheroids were found in small gray or yellow mucoid colonies—these latter often mottled with flaky material, edge irregular, lacy or raised (pl. A, fig. 3). The word pleomorphic is a term describing a wide variety of cellular shapes. There was no constant correlation, however, between the colony type—rough or smooth—and distinctive cellular morphology, the character of the medium and age of the culture. Under no experimental conditions were the individual cells of either rough or smooth colonies of streptococci entirely non-pleomorphic.

Biochemical characteristics

None of the strains hemolyzed 5 per cent sheep or human blood agar, but several strains hemolyzed 5 per cent rabbit blood agar when tested by the Brown method. All strains were insoluble in a 10 per cent aqueous solution of “Difco” bile.

Fermentation of carbohydrates was tested in 1 per cent filtered Pfanstiehl sucrose, lactose, salicin, raffinose, inulin and mannitol added to sugar-free veal infusion broth, pH 7.8, indicator brom-cresol-purple. Some of the more complex carbohydrates were utilized very slowly. All of the strains fermented sucrose; none of them utilized mannitol. One non-lactose fermenting rough strain was found. Of the 62 strains, 33 (53 per cent)

fermented salicin; 44 (70 per cent) fermented raffinose; 19 (30 per cent) fermented inulin and of these 19 strains, 4 also fermented salicin and raffinose.

Proteolytic action was studied in Loeffler serum by the test of Wollman, Urbain and Ostrowsky (1922). A day-old culture of streptococci in a liquid medium containing serum was successively inoculated with *E. coli*. The latter organism produced indol if the albumin of the medium had been utilized by the streptococci. There was no evidence of proteolytic action with either medium.

The test of Greenthal (1930) was used to detect the reduction of nitrates to nitrites. A few crystals of nitrite-free sodium nitrate were dropped in an eighteen to twenty-four hour broth culture of streptococci. A few drops of 10 per cent sulphuric acid, potassium iodide and dilute starch were added in succession. None of the pleomorphic streptococci thus tested gave a positive reaction.

As the fresh strains were isolated from the throat cultures they were used as antigens in agglutination tests with stock rabbit antiserum against the strain 42X (rough). Sixteen strains agglutinated by a serum dilution of 1:128 or more were selected for later work.

The mediums and the conditions evoking variation

First, many experiments were performed to determine the effect of a wide variety of mediums and conditions of growth in effecting variation in rough and smooth strains of pleomorphic streptococci. Second, when morphological variants of one form or another were found in a culture, single cell isolations were made to secure strains for more detailed experiments. Space forbids a complete account of all experiments whether effective or not; therefore only a concise summary of apparently successful procedures and results will be given.

The mediums used were:

1. Heated or hemolyzed sheep blood agar containing 2 per cent Witte peptone.
2. Veal infusion broth (double strength), pH 7.8, without salt, or peptone. (This is referred to subsequently as veal infusion broth no. 1).

3. Veal infusion broth no. 1 with 0.5 per cent mannitol.
4. Veal infusion broth no. 1 with 10 or 30 per cent anti-streptococcal rabbit serum with a demonstrable titer against homologous or heterologous strains.
5. Five per cent soluble potato starch in Tyrode's solution.
6. Cultural association with *L. acidophilus* in veal infusion broth no. 1 with 0.5 per cent glucose.

Method

The method found most generally satisfactory was as follows: selected strains (3 smooth strains and 3 rough strains) were transferred from fresh preliminary cultures on hemolyzed blood agar to a series of the test mediums. These were incubated at 37°C. for twenty-four hours, then at room temperature thereafter for twenty days. Smears and transfers to hemolyzed blood agar plates were made daily from each tube. Uninoculated tubes of medium were treated similarly as controls.

Results

1. Transfers of smooth or intermediate colonies of streptococci from blood or glucose broth to 2 per cent Witte peptone hemolyzed blood agar resulted in *rough*, proteolytic, feebly green-producing colonies containing bulging and constricted pleomorphic rods. These rods were impossible to stabilize on any medium and, particularly, in broth, and reverted quickly to the streptococcal form. Serial transfer on 2 per cent Witte peptone blood agar produced a smooth-to-rough colony transformation. Chamot and Georgia (1925), Davis (1927), and McAlpine and Brigham (1925) agree that Witte peptone is markedly different in growth-promoting properties and in chemical composition from other widely used peptones. The latter workers report that Witte peptone is rich in protein nitrogen with a low content of non-protein nitrogen and amino nitrogen in contrast to similar analyses of other brands. This agar then, with a high content of Witte peptone and blood, may be considered an "over-rich" growth-promoting medium.

2. In veal infusion broth, pH 7.8, without salt, peptone, glucose or blood—a poorly buffered "starvation medium" with a relatively high surface tension—the streptococcal chains increased

rapidly in length and in pleomorphism of individual cells. After forty-eight hours there were branching chains (pl. A, fig. 4), lateral and transverse fission of the cocci with lateral budding and a "fading out" of the formed coccal elements in the chains (pl. A, fig. 5). After six to sixteen days the chains were long, frequently branched sheaths containing randomly disposed blue-staining material no longer in the coccal form (pl. A, figs. 5, 5a, 5b). Viable transfers from strains in this medium reproduced green-producing colonies containing either streptococci or diplococci. There was no change in the inoculated rough or smooth colony form.

In the broth just described combined with 0.5 per cent mannitol the streptococcal chains soon fragmented to 4 to 8 coccal portions. In older cultures (seventy-two to ninety-six hours) three-cell division, lateral budding and discrete diplococci were commonly found. In certain cultures large faintly staining globoid bodies appeared; these cultures after seven to ten days reproduced on transfer to solid mediums gray, non-green-forming colonies containing short polar-staining diphtheroid organisms. There was a gradual change from smooth and rough colonies to the intermediate colony type.

4. In veal infusion broth, pH 7.8, containing no other ingredients except one-third volume anti-streptococcal rabbit serum, certain strains of streptococci were reduced to cocci and diplococci in an amorphous mass containing tiny stainable granules (pl. A, fig. 6). In these cultures no chain formation was seen after seventy-two to ninety-six hours; nor were the large inflated bodies found. Transfers from such cultures after varying periods of incubation very occasionally produced mucoid yellow colonies containing metachromatic staining diphtheroid rods (pl. B, fig. 6) or frequently gray intensely green-producing colonies of Gram-positive flattened diplococci. In the heterologous strains affected by this medium the titer ranged from 124 to 512; the titer against the homologous strain was 1024. The rough strains of streptococci in this medium were unchanged in colonial appearance; the smooth colonies on later transfers were topped with raised yellow caps—a precursor of a smooth-to-intermediate colony transformation.

5. In the soluble starch-Tyrode medium the definitely rough strains were rapidly and progressively converted to smoother strains. The chains grew to a moderate length with little pleomorphism. Then the chains fragmented with later Y-formed chains. After ninety-six to one hundred and twenty hours incubation, diplococci were present in the smears, and transfers to hemolyzed blood agar resulted in many reactive greening or "bleaching" colonies of Gram-positive diplococci, scattered among colonies containing green-producing streptococci.

6. The *L. acidophilus* and streptococcal strains were grown separately in the veal infusion broth for twenty-four hours, then combined for subsequent incubation in a flask. Lateral budding and short chain formation were found in nearly all combined cultures after twelve to twenty-four hours incubation. Massed diplococci alone remained of the streptococcal chains after three to six days. Streptococcal colonies were found on transfer to the eighth day; colonies containing diplococci only appeared on the fourth to sixth daily transfer and continued in some strains to the twelfth daily transfer. The *L. acidophilus* colonies were ignored. This lytic action on the streptococcal strains was not due solely to the lactic acid or metabolic products of the associated growth, for filtrates of seventy-two-hour cultures of *L. acidophilus* alone added to several twenty-four-hour cultures of streptococci had no similar effect. This observation confirms a similar one made by Schiller (1914).

No organisms were found in the uninoculated tubes either in the smear or by transfer.

Summary

The primary object in these and many other experiments was to effect a definite change in morphology in a known streptococcal culture. When streptococcal strains were passed by rapid serial transfer on a single favorable medium, changes in colony form did occur, but the pleomorphism was moderate and no variants formed. When, however, streptococcal organisms were *aged* in an unfavorable or a very stimulating medium there often occurred atypical cell division, branching chains, "fading" of formed cocci, swollen deeply staining bodies at intervals in the

chain and terminally, lateral budding, and finally complete reduction of the chains to an amorphous mass containing diplococci and granules. Transfers from such *aging* cultures to mediums of a different physical and chemical state resulted in colonies differing from the streptococcal colony and containing distinct variant cells: a diphtheroid, a diplococcus or a spindle-shaped rod.

FILTERABLE FORM FROM STREPTOCOCCI

It is an interesting fact that the mediums and methods employed for preliminary cultivation by most of the workers reporting filterable forms of streptococci are precisely those known to stimulate variation.³

The evidence up to this point has indicated that the production of pleomorphic elements, atypical cell division, lateral budding, branching and the reduction of chains to an amorphous mass preceded the formation of variants from a streptococcal culture. The three mediums in which a rapid chain reduction was most conspicuous were selected for preliminary cultivation preceding filtration experiments.

Method

Both rough and smooth strains of streptococci on heated blood agar were composed of moderately long chains of organisms occurring typically in pairs of flattened diplococci. These varied irregularly from minute organisms to elongated, inflated elements often centrally constricted. All gradations of size

³ Ramsine (1926)—streptococcal toxin prepared in glucose sheep-blood broth and cultural association with a related strain of streptococcus in 10 per cent sucrose broth.

Sedaillan and Gaumond (1927)—guinea pig peritoneal exudate, also whole blood.

Hauduroy and Lesbire (1927)—peptone water.

Urbain (1927)—5 per cent soluble starch.

Rashkowska (1928)—Martin's broth with 20 to 25 per cent ascitic fluid.

Riccitelli (14)—ascitic fluid broth.

Hadley, Delves, and Klimek (1931)—passage in lithium chloride broth; also in pancreatin broth.

Evans (1932)—rabbit brain passage.

Richardson (1932)—rabbit brain or peritoneal passage.

and shape of cocci occurred in the unbroken chain. Here and there in the chain areas appeared not retaining the blue stain and which had the appearance of a sheath no longer containing rounded stainable cocci.

Six strains (3 rough and 3 smooth) of pleomorphic streptococci were each inoculated into 8 tubes of the following mediums:

1. Ten per cent 42XS immune rabbit serum with veal infusion broth No. 1.
2. Five per cent soluble starch in Tyrode's solution.
3. Cultural association with *L. acidophilus* in glucose veal-infusion broth.

At the end of 6, 12, 24, 48, 72, 96, 120 and 144 hours incubation, a tube of each series was examined by smear, then filtered with a saline suspension of a fresh culture of *B. prodigiosus* as control with an equal amount of uninoculated medium. On alternate days as a further control, incubated but uninoculated mediums were also filtered and incubated with the test filtrates. The cultures containing *L. acidophilus* were controlled in addition by a separate filtration of *L. acidophilus* alone and incubated with the test cultures.

The filters were Seitz-Werke Germicide filters, Manteufel model, assembled without the narrow metal ring supplied by the manufacturers. The filtrate was received in a 10-by-1-inch tube placed in a liter filter flask. All filtrations were made with reduced water pressure, in most cases checked by a gauge. Serum-containing mediums were filtered at 100 to 120 mm. Hg pressure; the starch-containing medium was filtered at 150 mm. pressure. Filtrations often were completed in 4 minutes and always within twenty minutes. The large tube containing the filtrate was removed with aseptic precautions, plugged securely with a plug from a similar tube, covered with tinfoil and incubated at 37°C. for at least three weeks unless growth was indicated before that time.

Summarizing, filtration was performed when certain morphologic changes had occurred in the culture filtered. The entire filtrate was incubated in one tube and not dispensed to several kinds of medium. Additional similar medium equal in volume to

the filtrate was filtered with the culture to add to the nutritive properties of the liquid.

Results

The first evidence of growth in a filtrate under these conditions was not an opalescence, for the fluid remained clear, but a fine, barely perceptible precipitate which settled on the glass to the surface of the liquid. At first there was no precipitate in the bottom of the tube. As incubation continued the density of the precipitate increased yet the fluid remained clear. The first indication of a precipitate appeared between the eighth and twelfth days of incubation. When the tube on tilting presented a distinctly "ground glass" appearance it was opened for the *first time*.

The precipitate was always adherent to the glass and could not be shaken loose; it was most easily removed by scraping with a sterile Pasteur pipette with which a generous amount was transferred. Secondary growths from the clear liquid were never obtained; if at all, they were secured from transfers of the adherent precipitate.

About 0.1 cc. of the loosened precipitate was discharged from the pipette to the surface of a plate of 2 per cent Witte peptone heated-blood agar, and spread over a small area in the center of the plate. Twenty-four hours later 1 cc. of glucose broth was transferred to the same area, the moistened surface scraped with a loop, and the liquid re-transferred to another similar plate *seriatim* until a green reaction on the medium or colony formation indicated growth.

Usually after 5 or 6 serial passages there was a faint green reaction and when growth was definitely established the organisms "yellow-bleached" the medium. In smears from primary subcultures the organisms were diplococci often of uneven size. In stabilized cultures the organisms were diplococci, strongly Gram-positive, with flattened apposed sides.

The salient data derived from the filtration experiments were:

1. Diplococci were obtained from filtrates of cultures in

a. Immune serum broth: (1) from the homologous smooth strain; (2) from an heterologous rough strain.⁴

b. Starch-Tyrode medium: (1) from one smooth strain; (2) from two rough strains.

c. L. acidophilus association: from two smooth strains.

2. Viable elements appeared in the filtrates of those cultures *only* which were reduced to an amorphous state or a diplococcal form *before* filtration. This required a preliminary incubation of ninety-six to one hundred and forty-four hours.

3. The filtrates of cultures retaining the streptococcal form to the moment of filtration were sterile.

4. The filtrate upgrowth was slow (eight to twelve days) and had a marked attraction for glass—a characteristic in no way resembling cultural growth preceding filtration.

5. The organism recovered from the filtrate did not resemble the streptococcus cultured before filtration in morphology or biologic characteristics.

6. Control organisms *filtered with* the test culture did not pass the filter.

SINGLE CELL ISOLATION

In the technical manipulations recorded, the question may be raised whether the organisms described—the spindle shaped rod, the diphtheroid and diplococcus—were real variants of streptococci or were mere contaminants. While the cultural experiments were in progress it became equally necessary to develop a technic for the isolation of single cells for more detailed study. After preliminary trials, the technics advocated by Gee and Hunt (1929), and by Avery and Leland (1927) were combined. The chamber and technic described by the first authors were used to isolate the single cells; the chamber of the second authors was used as an incubating chamber.

This chamber was a brass ring 35 by 3 by 1 mm. cut from a brass tube, with short arms attached. On one side the chamber was completed by a 45- by 50-mm. No. 1 cover slip ruled into

⁴ From a third rough strain a diphtheroid was obtained from the filtrate after 12 serial passages.

minute squares (after paraffining) with a fine needle and hydrofluoric acid. The ring was dipped into hot paraffin and placed on a sterile etched slip in a petri dish. Witte heated blood agar, clarified while hot, was added to the well by pipette.

Single organisms were transferred by micro pipette (cf. Gee and Hunt, 1929) to the agar and the chamber completed and sealed firmly by dipping the free rim in hot paraffin and inverting on a 3- by 1-inch slide. The chamber was examined immediately with the oil immersion objective, the presence of single cells verified and their location plotted by means of the ruled squares. After incubation transfer was made from discrete colonies.

The swollen rods from proteolytic colonies and the diphtheroids from yellow mucoid colonies were not easily miscible in water, saline or broth. Shaking such cells in Tyrode's solution freed enough for isolation purposes. The diplococci were readily dispersed in broth. The spindle-like rods and the diplococci had a vitality as single cells far exceeding that of the diphtheroids. Of attempts to isolate cells from 18 diphtheroid strains only 7 single-cell diphtheroid strains were obtained on the Witte blood agar.

The spindle shaped rod

The unstable bulging and tapered rod was repeatedly found in primary transfers from the throat to heated or hemolyzed blood agar. In established streptococcal cultures the rod appeared in transfers from aging cultures on Loeffler serum or from blood broth to 2 per cent Witte peptone heated-blood agar. When the bulge or node was most evident there was also to be found (preferably by carefully made and stained contact preparations) a lateral "budding" from the nodular portion of the rod. Two or even three "buds" may be seen, each attached to the rod by a fine stalk (pl. B, fig. 1).

A rough strain of streptococci after six days incubation in glucose blood broth at room temperature was transferred to clarified 2 per cent Witte peptone heated-blood agar. Rough colonies developed interspersed with extremely minute colonies

the latter colonies detectible only with a long focus binocular colony microscope (pl. B, fig. 2). A contact preparation made by pressing a sterile cover slip on the growth was fixed in formol-Zenker solution, then dehydrated and stained with the Churchman modification of the Gram stain. The organisms in some of the larger rough colonies were spindle shaped rods with lateral budding (pl. B, fig. 3). In other colonies the rods were nodular and elongated containing granules arranged in pairs (pl. B, fig. 4). The organisms in the minute colonies were indefinitely stained granules and rods (pl. B, fig. 5). The organisms in the rough colonies quickly reverted to streptococci in broth cultures; the organisms in the minute colonies after 8 subcultures in semi-solid buffered brain agar, one transfer on Loeffler serum and finally in glucose broth became streptococcal in form.

The large, rod-like form (in massive and in single cell cultures) quickly merged into streptococci in liquid or on solid substrates. The development of the rod to the streptococcus was as follows:

A. The spindle-shaped rod, sharply tapered at either end, often constricted in its widest portion. At the pointed ends minute cocci appeared, which, dividing by simple fission, started a chain formation.

B. The nodal rods showed a regularly dispersed metachromatic stained condensation of chromatin alternating with deeply stained nodes. The stained granules lying in pairs side by side in the sheath appeared to round up as cocci. These dividing by simple fission formed a chain of pleomorphic streptococci.

The rod stage could not be stabilized, but while in this stage the organisms were definitely proteolytic on solid mediums. They had little or no effect upon blood pigments. There was no demonstrable change in sugar utilization or in nitrate reduction from that found in the corresponding streptococcus.

Cornil and Babes (1890), Arloing and Chantre (1894), Krasowska and Nitsch (1918), Brosq et al. (1923), Urbain (1927) and Euler (1927) observed such rod-like forms in primary cultures from body membranes or fluids in mediums containing blood or serum. All of these workers commented upon the fact that in such cultures the organisms within twenty-four to forty-eight

hours elongated with nodal swellings and evolved into typical streptococci. Other observers of this form in streptococcal cultures have recorded the branching of the streptococcal chains either preceding or following the development of the rod stage (Seitz (1896), Stolz (1898), Lorenz (1909), and Fry (1919)).

It is possible that the pleomorphic rod under discussion is similar to the "spore producing" rod of Evans (1926; 1927) isolated from encephalitic virus material or virus-inoculated brain emulsions in cooked meat medium, and more recently described as evolving from streptococci (Evans, 1932). It must be added, however, that the motility of the rods and the unmistakable spore formation described by Evans were not observed.

The diphtheroid

The sequence of colonial and morphologic change observed in the origin of a diphtheroid from a single-celled (growth from three connected coccal elements) rough strain of streptococci, No. 42XR, follows:

A transfer of the strain to veal infusion broth containing one-third volume of immune serum against a related strain, No. 389, was incubated at 37°C. for twenty-four hours and at room temperature for ten days. A transfer to hemolyzed blood agar produced discrete rough colonies interspersed with minute yellow mucoid colonies. The organisms in the mucoid colonies were large irregular coccal bodies, some of which seemed to contain two rods in a faintly staining "shell" (pl. B, fig. 7).

Two uninoculated tubes of the serum broth treated as the test culture gave no evidence of growth.

The yellow mucoid colony on serial transfer produced no green reaction on hemolyzed blood agar (pl. B, fig. 6). The smear was composed of metachromatic-staining curved and clubbed rods (pl. B, fig. 8). A return from this rod to the streptococcal stage was obtained by alternate transfer on fresh Loeffler serum with condensation water and glucose veal-infusion broth, forty-eight to seventy-two hours elapsing between transfers. This method of the alternate use of solid and liquid mediums, with a prolonged period between transfers, was the most successful method used in

inducing the diphtheroid strains to show streptococcal relationships.

Diphtheroids in gray colonies repeatedly occurred after prolonged incubation of streptococci, usually at room or icebox temperatures, followed by transfer to a different medium. In five instances such gray diphtheroid-containing colonies resulted in transfers to a dissimilar medium from aged single-celled strains of diplococci (of streptococcal origin). The yellow mucoid type of colony appeared in transfers from prolonged incubation in a serum-containing liquid medium or in primary transfers from the throat.

The diphtheroid appeared to arise from streptococcal or diplococcal antecedent cells by an enlargement of certain cocci in which chromatin possibly was reorganized, reproducing upon transfer as rods. As these rods merged into the streptococcal form the metachromatic-staining material first condensed at certain points in the rod, then rounded up as cocci which reproduced as streptococci by simple fission.

The biochemical characteristics of such diphtheroid strains in general were: a limited and slow utilization of sucrose, lactose, maltose and dextrin; no effect upon blood pigment; moderate proteolytic action (yellow strains); and slight reduction of nitrates to nitrites (especially chromogenic strains).

Investigators who have reported diphtheroids in known streptococcal cultures have used for the most part body fluids or mediums containing such fluids (Lemoine (1896), Mellon (1917), Urbain (1927), and Ramsine (1927)). The diphtheroids occurred only after considerable aging of the streptococci in such mediums.

Jensen and Morton (1931) isolated a streptococcal strain which on blood agar was consistently diphtheroidal in form and in glucose brain-broth as constantly grew as streptococci. The two phases were found to differ in virulence, cataphoretic potentials, peroxide production and serologic specificity. Koch and Mellon (1930) support the view that diphtheroids from blood cultures may at times be the attenuated, non-virulent phase of virulent streptococci. Thompson (1932), however, in a large

series of blood cultures found no evidence of a genetic relationship between diphtheroid and streptococcus.

The diplococcus

As a variant form in streptococcal cultures the diplococcus appeared to take form in two ways: directly by lateral budding from cocci in the intact streptococcal chain; and indirectly, following more or less complete disintegration and dissolution of the chains.

The formation of diplococci indirectly in liquid mediums was preceded by a branching of the chains, then a slow, cumulative

TABLE 1

MEDIUMS	INCUBATION PERIOD	MORPHOLOGY
1. Heated blood agar.....	6 days	Diplococci
2. Veal inf. broth, pH 8.2, 0.5 per cent glucose.....	96 hours	Diplococci
3. 2 per cent Witte peptone blood agar, no salt....	48 hours	Diplococci
4. Ascitic fluid broth, pH 8.0.....	72 hours	Diplococci
5. Ascitic fluid agar, pH 7.8.....	48 hours	Huge and minute cocci
6. Loeffler's serum.....	48 hours	Clubbed rods
7. Hemolyzed blood agar, 2 transfers.....	48 hours	Slight green reaction
8. Veal inf. broth, pH 7.8, 0.5 per cent glucose.....	24 hours	Streptococci

fading of the formed coccal elements. The randomly-dispersed blue-staining material for a time retained the stain within a faintly pink-staining streptococcal sheath and then the chain formation completely disappeared (pl. A, fig. 5a). Following this, smears revealed only Gram-positive cocci and diplococci in a pink amorphous mass containing minute faintly-staining granules (pl. A, fig. 6). *This granule formation is stressed.* Transfers from this terminal stage to solid mediums frequently produced diplococci only and not streptococci.

Filtration, if performed after the terminal granular stage had been reached, was useful in separating viable elements which on subculture resulted usually in diplococci and, less often, in diphtheroids. Such diplococci obtained from cultures and filtrates

were single-celled and resultant growths were carried in stock on heated blood agar.

Two single-celled filtrate strains of diplococci, 42XS-d, derived from a filtrate of No. 42XS in homologous immune serum broth, and 389-d, derived in a similar manner from homologous immune serum broth, were cultured to determine whether such diplococci were truly related to the original streptococci. The 42XS-d diplococcus returned to the streptococcal form after transfers through the sequence of mediums shown in table 1.

The recovered streptococcus fermented sucrose, lactose and salicin completely (pH 5.4) and raffinose slightly (pH 6.0); the original streptococcus failed to ferment raffinose but fermented the other sugars. The original streptococcus was agglutinated by its homologous antiserum in a dilution of 1:1024, the recovered streptococcus was agglutinated by this serum in a dilution of 1:128; there was no agglutination of the intermediate diplococcus.

The diplococcus 389-d after aging twenty-two days in mannitol broth was incubated seventy-two hours on a hemolyzed blood agar (made with a filtrate of a broth culture of the streptococcus 389). The organisms in the flat, concentric, feebly green-producing colonies were huge and minute diplococci. Some of the larger cocci appeared to be budding (pl. A, fig. 7). Two transfers on 2 per cent Witte heated-blood agar followed by veal infusion glucose broth produced the streptococcal form.

The original streptococcus, No. 389, failed to ferment salicin and mannitol, the recovered streptococcus failed to ferment mannitol. The antiserum homologous for the original strain No. 389 agglutinated the organisms in a dilution of 1:512; the recovered streptococcus was agglutinated in a dilution of 1:64; the diplococcus was not agglutinated.

The diplococcus (of streptococcal origin) possessed two striking biologic characteristics:

1. The diplococcus had a far more pronounced action upon blood than the streptococcus; it "yellow-bleached" blood agar whereas the streptococcus on the same plate produced a moderate narrow green zone.

2. The diplococcus in liquid mediums strongly reduced nitrates to nitrites. This property was not possessed by the corresponding

streptococcus. The diplococcus was not bile-soluble and feebly fermented five of the six sugars tested.

The essential points in the literature of pleomorphic streptococci over three decades were tabulated in abstract by Donaldson (1922). Many strains when first seen on artificial mediums were diplococci, and the term "diplo-streptococcus" was a common characterization of pleomorphic streptococci.

Löhnis (1921) emphasized the stability of variant cocci and diplococci from streptococci and other bacterial species. Once stabilized by various technics the organisms grew readily and were resistant to change. The same point was re-emphasized by Hauduroy (1929) and by Hadley and collaborators (1931). Nevertheless, the transformation of diplococci *from* streptococci back *to* the streptococcal stage has been recorded by Lorenz (1909), Taddei (1909), Mellon (1911), Euler (1927), and Lautier (1929).

ANTIGENIC RELATIONSHIP OF STREPTOCOCCAL AND VARIANT STRAINS

The low-titered agglutination of the diphtheroid variant by rabbit serum prepared against the undissociated streptococcus and the total absence of agglutination of the variant diplococcus by this serum indicated the need for a more complete record of the serologic interrelationship of the three forms: streptococcus, diphtheroid and diplococcus.

Rabbits in duplicate were immunized with each of the following antigens representing two distinct strains:

- | | | |
|---|---|--|
| A | { | Rabbits (2) immunized with the original rough streptococcal strain No. 389. |
| | | Rabbits (2) immunized with No. 389 diphtheroid from an eight-day culture in mannitol broth. |
| | | Rabbits (2) immunized with No. 389 diplococcus from a filtrate of streptococcus No. 389 grown in homologous immune serum. |
| B | { | Rabbits (2) immunized with original smooth streptococcal strain No. 42XS. |
| | | Rabbits (2) immunized with 42XS diphtheroid (yellow mucoid) from a transfer from a forty-six-day blood broth culture to heated blood agar. |
| | | Rabbits (2) immunized with 42XS diplococcus from a filtrate of No. 42XS in homologous immune serum broth. |

389 series

Agglutination tests were set up between each antigen of the No. 389 series and each immune serum, controlled by similar tests with normal serum of the immunized rabbits and the usual saline controls. The antigens in the control tests were not agglutinated.

42XS series

A similar test was made with the antiserums of the 42XS series of antigens. Two sets of the serial dilutions of serums were prepared; to one set were added living antigens washed from

TABLE 2

Agglutinations of strain No. 389, and its variants by their respective antiserums

ANTISERUM AGAINST	ANTIGEN TESTED	AGGLUTINATION FINAL SERUM DILUTION
389 streptococcus	389 streptococcus	1,024
	389 diphtheroid	64
	389 diplococcus	None
389 diphtheroid	389 diphtheroid	512
	389 streptococcus	32
	389 diplococcus	64
389 diplococcus	389 diplococcus	1,024
	389 diphtheroid	32
	389 streptococcus	None

twenty-four-hour agar cultures with 0.8 per cent NaCl solution. To the other set were added stock antigens used for immunization and diluted with saline to the required turbidity.

The antigens in the control tests were not agglutinated. The diphtheroid antigen appeared to be partially lysed in the first 3 or 4 tubes of the dilution series by both the streptococcal and diplococcal antiserums. The agglutinative titers with the diluted stored antigens of the same series were similar except that the seeming lytic effect against the diphtheroid antigen by the streptococcal and diplococcal specific serums was not evident.

In these and similar tests the diphtheroid antigen was agglutinated not only by its homologous antiserum, but also to some degree by the serums against the streptococcus and the diplococcus. Both the streptococcus and the diplococcus were agglutinated perceptibly by the diphtheroid antiserum.

It thus appears from the evidence of the serologic tests that the diphtheroid may be a form intermediate in antigenic structure between the streptococcus and the diplococcus. The sequence in morphologic changes in cultures also indicated this relationship. When streptococci were derived from diplococci they passed

TABLE 3

Agglutinations of strain No. 42XS and its variants by their respective antisera

ANTISERUMS AGAINST	ANTIGEN TESTED	AGGLUTINATION FINAL SERUM DILUTION
42XS streptococcus	42XS streptococcus	1,024
	42XS diphtheroid	128
	42XS diplococci	None
42XS diphtheroid	42XS diphtheroid	512
	42XS streptococcus	64
	42XS diplococcus	32
42XS diplococcus	42XS diplococcus	1,024
	42XS diphtheroid	64
	42XS streptococcus	None

almost without exception through an intermediate diphtheroid stage before again merging into the streptococcal form. The one exception to this generalization was a diplococcus from a filtrate growth which on subculture merged directly into a streptococcus by a preliminary lateral budding preceding active simple fission (pl. A, figs. 7, 8, 9).

DISCUSSION

The pleomorphic green-producing streptococcus removed from its natural habitat—the living body—is composed of flattened diplococci in chains interspersed with enlarged, constricted bodies.

Huge cocci, diplococci and rods, both spindle-shaped and diphtheroid, complete the varied microscopic picture.

The streptococcus as well as three of the morphologic elements—the diplococcus, the diphtheroid and the spindle-shaped rod—occurred discretely in separate culture. The spindle-shaped rod was formed especially on solid mediums favoring growth; its existence as such was brief for it quickly elongated by fission to a chain. A prolonged incubation of streptococci in mediums limiting growth and multiplication, followed by transfer to mediums of a different physical state and chemical composition, produced variant colonies containing variant cells—the diphtheroid or the diplococcus. Once formed these cells were maintained by rapid transfer on the same mediums on which they arose.

Visible changes occurred in the streptococcal cultures preceding the terminal growth of variants. Atypical cell division produced branched chains or discrete cocci; the chain sheath disintegrated and finally cocci and granules only remained of the original culture. Granule formation was always present in liquid cultures in which, after filtration, a secondary growth developed. The diplococci recovered from such filtrate growths may have originated from (1) diplococci passing by chance a faulty filter or (2) few or many potentially coccal forming granules.

One-half of the volume of the incubated filtrate contained the metabolic products of growing and dying organisms; the remainder—fresh medium filtered with the culture—presumably furnished sufficient nutriment to permit the development of a filter passing form. It cannot be denied that since diplococci were present in the medium before filtration certain organisms may have passed the filter and slowly reproduced themselves. Against this argument may be placed these observations: (1) in filtering simultaneously diplococcal and granule-containing cultures with *B. prodigiosus* in no instance was there a re-growth in the filtrate of the control organism; (2) the filtrate growth developed very slowly and had a marked attraction for glass—a characteristic quite unlike that of broth cultures of single cell diplococci which produced a turbid growth in forty-eight hours; (3) repeated sub-

culture on solid mediums was required before the filtrate growth assumed the diplococcal form and attributes.

The granule may be merely a lifeless product of bacterial metabolism or it may be a specialized physiologic unit carrying in the most condensed form the latent characteristics of the strain. Löhnis (1921) compiled many observations of granule formation by bacteria and discussed the phenomenon in relation to regeneration of the usual bacterial cell. Hadley et al. (1931) in agreement with the interpretation of Löhnis, recently, discussed further the possible relation of granule formation to filter passing forms of the Shiga bacillus and related species. Kendall (1931; 1932) cultured several species of bacteria in a special protein medium. He examined his cultures with the ultramicroscope immediately before and after filtration and found granules which he interpreted as living filterable forms. However formed, the diplococcus from streptococcus filtrate growths possessed biochemical functions quite distinct from those of the streptococcus cultured for filtration. Repeated changes in environment induced in two strains of filtrate diplococci morphologic variations resulting finally in streptococci similar in attributes to the original streptococcal strains.

The chief interest of the cultural results lies in the evidence, under the conditions studied, of the range of variable form and function by which streptococci perpetuate themselves. Further study is suggested in regard to chemical analyses of the antigenic constitution of the original and variant organisms, and observation of their effect upon living cells by animal inoculation or by combining them with normal and specifically immunized tissue cultures.

CONCLUSIONS

1. Rapid serial transfers of rough or smooth green-producing streptococci upon a single medium induce a change in colony type but *no change* in morphology. Streptococci aged in unfavorable or in stimulating mediums followed by repeated transfers to mediums of differing physical and chemical states produce variant colonies containing morphologically variant cells.

2. Pleomorphic green-producing streptococci give rise by described technics to three variants—a blood-bleaching, nitrite-forming diplococcus, in rounded, moderately rough colonies, a diphtheroid in gray or yellow mucoid colonies, and a proteolytic spindle-shaped rod in definitely rough colonies.

3. The diplococcus and the diphtheroid possess an antigenic structure and biochemical functions differing from each other and from those possessed by the original streptococcus. Diplococci and diphtheroids so formed give rise again to streptococci by a process which is preceded and probably depends upon a changed mode of cell division. The streptococcus thus recovered resembles, but is not identical with, the original streptococcus in serological properties and biochemical action.

4. A precipitate containing viable forms occasionally develops in the filtrates of streptococcal cultures. This occurs when atypical cell division, chain reduction and granule formation is clearly evident in the culture before filtration.

5. The viable forms eventually by a suitable technic assume a visible form, in most cases a diplococcus, similar to the diplococcus isolated from the same culture before filtration. In two cases diplococci from the filtrates were converted to streptococci with attributes similar to but not identical with those of the original streptococcal strains.

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PLATES

PLATE A

FIG. 1. Intermediate colony. $\times 100$.

FIG. 2. Markedly rough colony. $\times 150$.

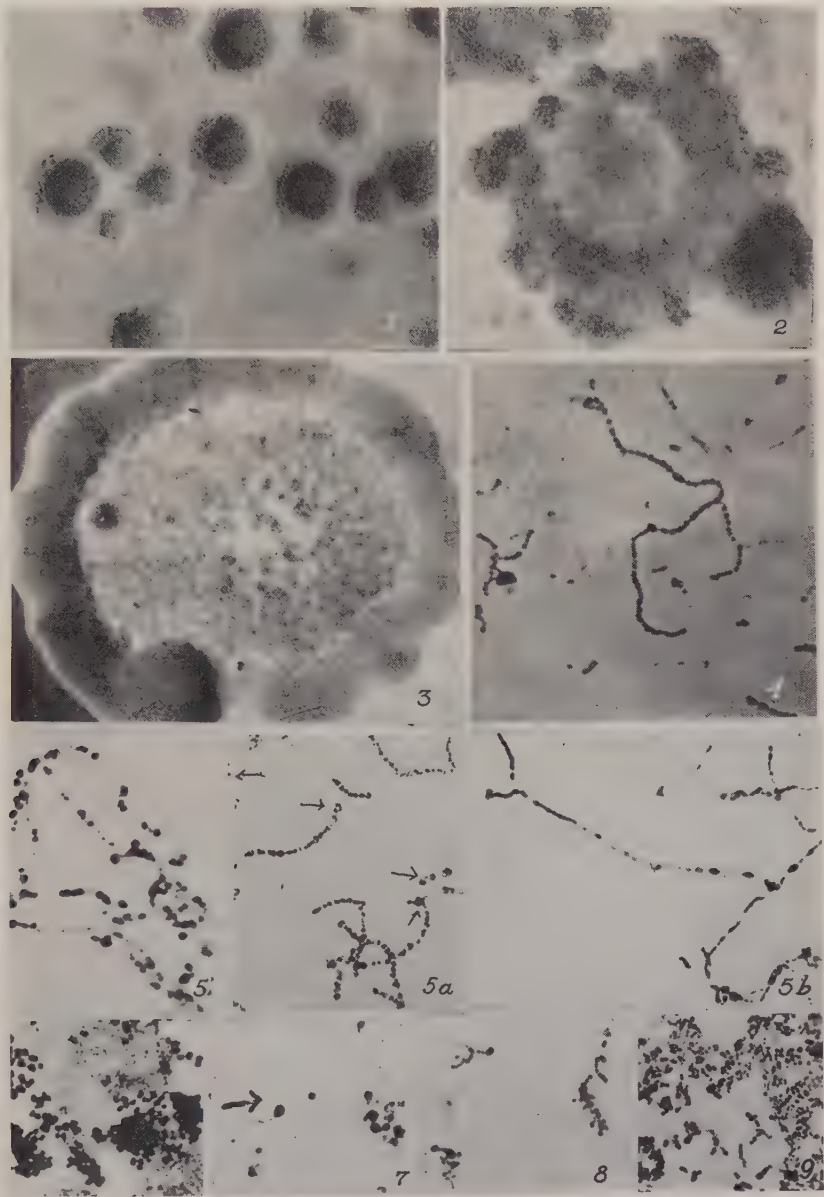
FIG. 3. Mucoid colony flecked with yellow material containing diphtheroids. $\times 200$. All colonies on hemolyzed blood agar, forty-eight hours.

FIG. 4. Rough strain in veal infusion broth, twenty-four hours. Note branching.

FIGS. 5, 5A AND 5B. Same culture after six to ten days. Streptococcal sheath still intact. $\times 1000$.

FIG. 6. Smooth strain in 10 per cent homologous immune serum broth six days. Diplococci and granular material. $\times 1000$.

FIGS. 7, 8 AND 9. Filtrate diplococcus. Lateral budding of cocci and transitional forms to streptococci. $\times 1000$.



(Ruth A. McKinney: Forms of Green-producing Streptococci)

PLATE B

FIG. 1. Contact preparation of culture on 2 per cent Witte heated blood agar, twenty-four hours. Lateral budding from swollen rods. $\times 2000$.

FIG. 2. Rough strain on clarified Witte blood agar; large and minute colonies. $\times 150$.

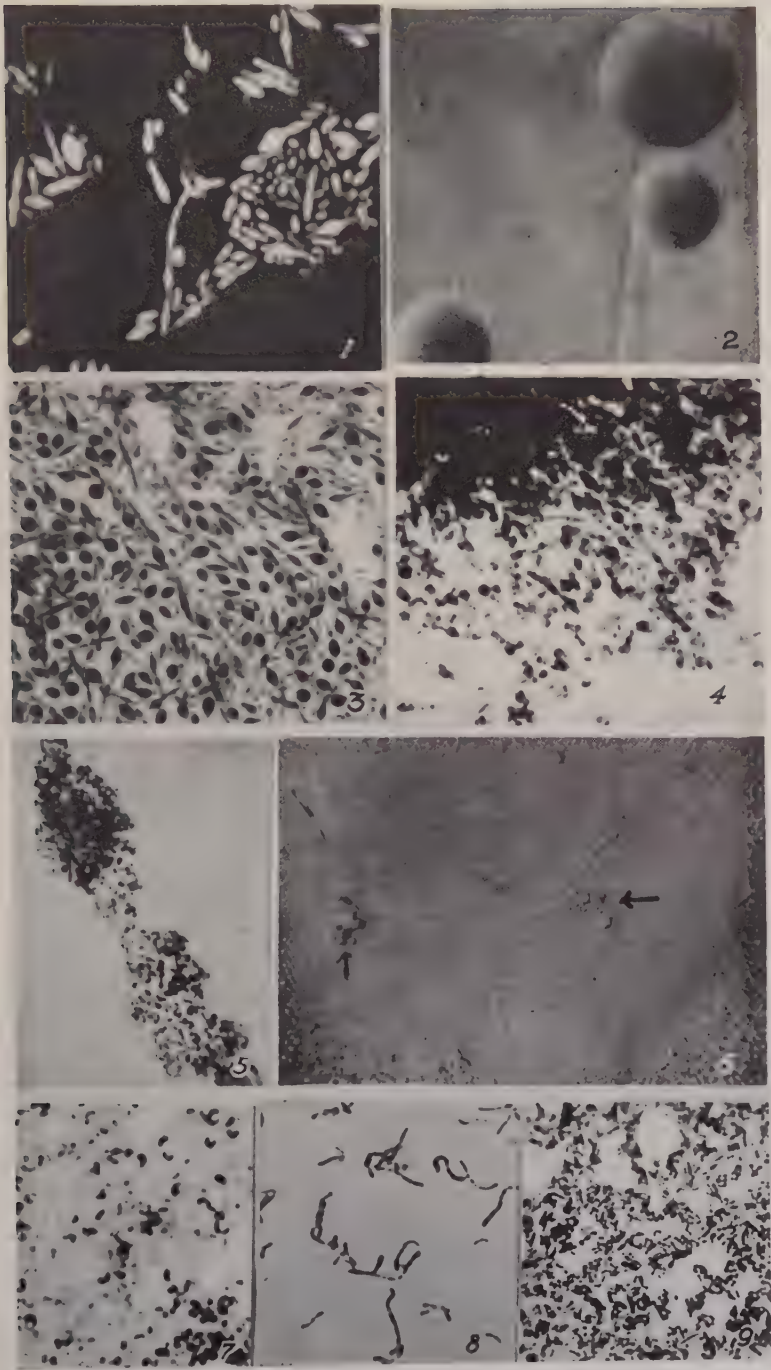
FIGS. 3 AND 4. Stained contact preparation of large colonies in figure 2. Spindle-shaped rods with lateral budding; nodular metachromatic staining rods. $\times 1200$.

FIG. 5. Stained preparation of minute colony, fig. 2. $\times 1200$.

FIG. 6. Yellow mucoid variant colonies from rough streptococcus. $\times 950$.

FIGS. 7 AND 8. Organisms in mucoid yellow colony, a variant of a rough streptococcus. $\times 1000$.

FIG. 9. Smear from other variant gray diphtheroid containing colony. $\times 1000$.



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